Studies on the effect of various elicitors for enhancing secondary metabolites production in *Piper longum* L.

Synopsis

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INTRODUCTION

In this modern era, the conceptualization of getting into the "roots" of medicines is gaining popularity. The advancements in science in concert with the knowledge of Vedas have enabled the analysis of different compounds from the endemic plants and identifying new plant species used in various cultures for ages for its benefits (Salehi *et al*., 2019). For thousands of years, plants are being explored as a source of natural medicines, food, and spiritual purposes. The geographical position of Indian subcontinent provides varied climatic conditions and seasons that offer a positive contribution in the survival of numerous plant species with curative potentiality (Zaveri *et al*., 2010). These plant-derived drugs have been found as the cogent source to cure severe diseases with greater safety profile and efficacy (Chauhan *et al*., 2011). One of the extensively ranged plant genera of the pantropical regions is *Piper* (Piperaceae).

Piperaceae "Pepper family"

Family Piperaceae includes 4000 species divided into 5 genres of which *Piper* (2000 species) and *Peperomia* (1600 species) are quite significant. Economically, commercially, and medicinally *Piper* species are crucial (Yadav *et al*., 2020). Owing to its third-place *Piper longum* (Piperaceae) is an economically important species of the genus after *Piper nigrum* (The king of spices) and *Piper betel* (Rani and Dantu., 2011).

Piper longum ("*pippali*" or long pepper) is sometimes called Javanese, Indian, Indonesian long pepper, is a flowering vine commonly cultivated for its fruit used as a spice (Kumar *et al*., 2011). It is distributed worldwide in tropical and subtropical regions, the Indian subcontinent, Sri Lanka, Middle Eastern countries. The plant is considered native to South Asia and the hotter parts of the Indian subcontinent from central to the northeastern Himalayas. It can be cultivated or grown in the wild (Zaveri *et al*., 2010).

P. longum is a slender, aromatic, diecious, perennial shrub with woody roots and numerous creeping jointed stems thickened at node spreading on the ground. The leaves are 5-7cm long, ovate, cordate, sub-acute, entire, alternate, exstipulate. The plant bears unisexual flowers that grow in solitary erect spikes during or just after the rainy season (Chauhan *et al*., 2011). Male spike is usually longer and slender whereas female spike is 1.3–2.5 cm long and 4–5 mm diameter. The fruits are ovoid present in

fleshy spikes, turn from green to black when gets matured. For commercial intent, the mature spikes are collected and dried as “pippali”, and the root radix known as “pippalimula” comes in 3 grades (Gani *et al*., 2019).

Secondary metabolites are bioactive compounds synthesized during the primary metabolism of plants. Their primary role in plants is defense against pathogens, insects, herbivores, or others. The biotic and abiotic stresses induce biosynthesis of secondary metabolites as phytoalexins, hypersensitive responses and structural defensive barriers, such as lignin deposition on the cell wall, among other stresses (Singh and Dwivedi., 2018). Molecules that trigger a response in a living organism resulting in the accumulation of secondary metabolites are termed as "Elicitors". They can be derived from various sources, biotic or abiotic, as well as physical factors. Elicitors are effective means for improving the production of plant valuable compounds (Vasconsuelo and Boland., 2007).

Plants are potent "non-polluting chemical factories" as they synthesize enormous varieties of biologically active compounds. Chemical industries make tremendous efforts to synthesize these compounds, but the triumph is limited due to constraints. Despite the advances in plant biotechnology to produce these pragmatic metabolites, the levels are not adequate to fulfil commercial applications and prerequisites. Genus *Piper* is identified for its remarkable production of various classes of secondary metabolites especially those derived from “Shikimate Pathway” such as phenylpropanoids, flavonoids, lignans, chalcones, neolignans (Lago *et al*., 2012).

Different plant parts of *P. longum* revealed the presence of various important bioactive compounds viz. alkaloids, flavonoids, glycosides, tannin, phenol, essential oils, and sterol (Zaveri *et al*., 2010). The fruit contains many alkaloids and related compounds, the most abundant of which is piperine, followed by methyl piperine, pipernonaline, piperettine, asarinine, pellitorine, piperundecalidine, piperlongumine, piperlonguminine, retrofractamide A, pergumidiene, brachystamide-B, N-isobutyl decadienamide, brachyamide-A, brachystine, pipercide, piperderidine, longamide, dehydropipernonaline piperidine, and tetrahydro piperine (Chauhan *et al*., 2011; Gani *et al*., 2019). Piperine is the major and active constituent of long pepper. The piperine content is 3-5% (on dry weight basis). Piperidine is used as a marker compound in *P. longum*.

The root (Pippalimula) and fruit (Pippali) are of high sanative relevance. According to the Ayurveda system of medicine, roots are pungent and having heating, stomachic, laxative, anthelmintic and carminative properties. It improves the appetite and is constructive in bronchitis, abdominal pain, disease of the spleen, and tumors. According to the Unani system, root has a bitter, hot, and sharp taste and is used as carminative, hepato-protective, stomachic, abortifacient, haematinic, diuretic, digestive. It also cures liver inflammation, joint pain, lumbago, snakebite, scorpion-sting, and night-blindness. Pippalimula is reported to have analgesic, antifungal, antimicrobial and antioxidant activities. Also, the fruit and root extract of *Piper longum* along with ciprofloxacin showed good synergistic activity against Methicillin-resistant Staphylococcus aureus (MRSA) (Joshi *et al*., 2013). It is widely used to cure diseases like leprosy and tuberculosis and is also used in the treatment of cough, dysponea, cardiac and spleen disorders, chronic fever, gout, rheumatic pain, etc (Choudhary and Singh.,2017).

The dietary piperine is known for its bioavailability and digestion enhancing properties. *In vitro* studies suggested the role of piperine in relieving oxidative stress by quenching free radicals and reactive oxygen species. It is known to act as an antimutagenic and antitumor agent. The root extract and piperine exert antioxidant activity and are protective in the myocardial ischemic condition (Jagdale *et al*., 2009). CNS depressant and analgesic activities of methanol extract obtained from the leaves of *Piper longum* (Vedhanayaki *et al*.,2003; Mamun *et al*., 2011). *Piper longum* was studied for the antifertility effect in female rats (Laxmi *et al*.,2006). It is most used to treat chronic bronchitis, asthma, constipation, gonorrhea, paralysis of the tongue, diarrhea, cholera, chronic malaria, viral hepatitis, respiratory infections, stomachache, bronchitis, diseases of the spleen, cough, and tumors (Kumar *et al*., 2011).

Pippali or *"Rasayana"* is used in Ayurveda treatment and numerous ayurvedic preparations are made from them such as 'Trikatu', Vardhamanas Pippali Rasayana, Talisapatradi Churna, Pippalyedyesava, Guda-Pippalyadi Choorna, Shiva gutika, Kaishore Guggulu, Kumarysava, Kanakasava, Balacaturbhadrika, Shringyadi churna, Amritarishta (also called Amrutharishtam) (Subramaniam *et al*., 2021).

Keeping in view the difficulties of extraction and threatening the existence of the plant these bioactive compounds are produced alternatively *in vitro* through plant cell/ tissue culture techniques, application

of metabolism-inducing factors or elicitations, controlling biological factors such as light intensity, applications of nanoparticles, and production through fungal endophytes. These techniques are used widely to enhance the production of these bioactive metabolites by interacting with the biochemical pathways of the plant either promoting or silencing the mechanism to produce them in high quantities (Alvarado *et al*., 2019).

"Elicitation" is a process of inducing or enhancing the synthesis of a secondary metabolite by the plants to ensure their persistence, survival, and competitiveness. Elicitors may be biotic as well as abiotic. Phytohormones such as Methyl Jasmonate (MeJA), Jasmonic acid (JA), Salicylic acid (SA) are used widely for the elicitation studies presenting their key roles in enhancing these valuable compounds of pharmaceutical importance (Singh and Dwivedi., 2018). Fungal endophytes are also a well-established source of these pharmacologically important plant-derived products. These compounds are synthesized with key precursors but with variations in the biosynthetic pathway and can be classified as non-ribosomal peptides, terpenes, and alkaloids (Singh *et al*., 2021).

The high stability and productivity of the hairy root cultures made it emerge as a valuable biotechnological tool for secondary metabolite production. Furthermore, these cultures can be subjected to elicitors for higher accumulation in both small- and large-scale production (Pistelli *et al*., 2010; Srivastava and Srivastava., 2008; Singh *et al*., 2018). The alteration in the biosynthetic pathway by these elicitors has paved a way to yield these valuable compounds in ample quantities which in turn will be implemented by pharmaceuticals, cosmetic industries, etc. Scrutinizing the literature had revealed that not much work had been done to escalate the production of secondary metabolites of *Piper longum* by elicitors.

OBJECTIVES OF THE STUDY

Secondary metabolite accumulation is persuaded by environmental factors but is modulated by the application of exogenous compounds. Considering the demands of these bioactive compounds in the field of the pharmaceutical industries the yield as well as quality in terms of plant metabolites in the green factories are needed to be enhanced and to fulfill this demand following objectives will be undertaken:

1. To determine the effect of different chemical elicitors on physio- chemical properties and secondary metabolite accumulation in *Piper longum*
2. To determine the effect of different biotic elicitors on physio- chemical properties and secondary metabolite accumulation in *Piper longum*
3. Determination of the effect of elicitors on the biosynthetic pathway of piperine

REVIEW OF LITERATURE

A method had been devised for the rapid and efficient isolation and identification of piperine from the fruits of *Piper longum* through melting point, HNMR, XRPD, mass spectral and molecular modeling (Mishra., 2010). The zonal variation effects the piperine content in roots of *P. longum*, among the agro- climatic zones considered Keonjhar region of Odissa, India reported the highest concentration of the piperine. Spectrophotometric and HPLC results showed that the content is highest in the plants grown through nodal roots followed by petiolar and apical when extracted from methanol (Mohapatra and Basak., 2015).

The remedial properties of *P. longum* are well known in the Unani system of medicine. The primary constituents isolated from the various parts are piperine, piperlongumine, sylvatin, sesamin, diaeudesmin piperlonguminine, pipermonaline, and piperundecalidine. These compounds showed immense therapeutic use including treatment of chronic bronchitis, asthma, constipation, gonorrhea, paralysis of the tongue, diarrhea, cholera, chronic malaria, viral hepatitis, respiratory infections, stomachache, bronchitis, and diseases of the spleen, cough, and tumors (Gani *et al*., 2019; Kumar *et al*., 2011; Chauhan *et al*., 2011).

Piper species have demonstrated strong efficacy against both malignant and non-malignant diseases. Piperine is a valuable alkaloid found in the fruits and roots of *P. longum* (long pepper), *P. nigrum* (black pepper) and stems of *P. retrofractum*. The results of ash and extractable values were comparable with the results in Ayurveda pharmacopeia of India. Phytochemical analysis reveals the presence of saponins, alkaloids, tannins, phenols, flavanoids, terpinoids, cardiac glycosides and steroids. Heavy metal concentrations were found to be within the limits. Results of these physico-chemical parameters, phytochemical, macroscopic and microscopic analysis can be used to assess the

quality and detection of any adulteration *for P. nigrum, P. longum and P. retrofractum* (Dahanayake *et al.*, 2019).

The phytochemicals and essential oils of *Piper* species have shown strong antioxidant activity, in comparison with synthetic antioxidants, and demonstrated antibacterial and antifungal activities against human pathogens (Salehi *et al*., 2019). The molecular basis of genotypic differentiation between male and female plants using RAPD and development of sex associated DNA markers have been discussed along with some medicinal and pharmacological properties of the spice (Manoj *et al*., 2004).

Piper nigrum contains an important bioactive compound piperine (2-7.4%) it consist of various pharmacological properties antiproliferative, antitumor, antiangiogenesis, antioxidant, antidiabetic, antiobesity, cardioprotective, antimicrobial, antiaging, and immunomodulatory effects in various in vitro and in vivo experimental trials. Furthermore, piperine has also been documented for its hepatoprotective, anti-allergic, anti-inflammatory, and neuroprotective properties (Haq *et al*., 2021).

Twenty drug like phytochemicals isolated from *P. longum* are reported to have immunomodulatory potency out of which 7 were found to be the potent regulator of 5 FDA approved drug targets and the multi targeting capacity of 3 phytochemicals were involved in neuroactive ligand receptor interaction pathway (Choudhary and Singh., 2018). The analysis of different parts of male and female plants pf *P. longum* reveals the presence of piperine in female spike, roots and leaves and in male the root extract is tested positive for the compound (Prasad *et al*., 2018).

The leaf oil of *P. longum* was rich with the phenyl propanoids apiole (50.0%) and myristicin (26.9%), whereas sesquiterpene hydrocarbons β-caryophyllene (28.6%), α-humulene (22.8%), and germacrene D (14.6%) were the major constituents in *P. chaba* leaf oil. In the fruit oil of *P. longum*, aliphatic compounds predominated with n-pentadecane (15.8%), whereas *P. chaba* fruit oil mainly had sesquiterpene hydrocarbons such as germacrene D (21.5%), β-caryophyllene (18.5%), and α-humulene (11.4%). In *P. longum* fruits, piperine content was lower (0.03%) compared to *P. chaba* fruits (1.32%). In *P. chaba*, the piperamides were mainly piperine, whereas other piperamides were predominant in *P. longum*. Antioxidant activity was higher for *P. longum* fruits as compared to *P. chaba* fruits (Rameshkumar *et al.*, 2011).

The *in vitro* propagation of *P. longum* was successfully established using different growth regulators such as benzyladenine, kinetin and picloram (Soniya and Das., 2002). A media supplemented with 1 mg/l 6- benzyladenine and 40mg/l ascorbic acid was used to reduce browning in shoots regenerated bythe petiolar explants of pippali (Rani and Dantu., 2011).

The essential oil production, yield of total volatile oil content in fruit and leaf was higher than that of root and stem. The principal components in root, stem, and fruit were comparable. The oil composition of root (62.0 %), fruit (71.5 %), and stem (69.2 %) was dominated by the presence of monoterpene hydrocarbons while the leaf had sesquiterpene hydrocarbons (68.7 %) as the major components (Varughese *et al*., 2016).

The comparative transcriptome analysis of *Piper longum* leaf, spike and roots provided gene families which are associated with the piperine biosynthesis. The differential expression of the 27 genes were confirmed by the qRT-PCR based upon the upregulation and down regulation of these genes (Dantu *et al*., 2021).

Effect of methyl Jasmonate and salicylic acid on secondary metabolite contents:

MeJA increased the total phenolic content without affecting the growth of *Castilleja tenuiflora* at 100µM accompanied with the increase in the PAL activity and H_2O_2 content. There was a considerable increase in the content of verbascoside and acubin content. The treatment resulted in the upregulation of the metabolite related genes s Cte-PAL1, Cte-CHS1, CteDXS1, and Cte-G10H compared to control (Rubio-Rodríguez *et al., 2021*).

In hairy root culture of *Ficus carica* L. the elicitation of MeJA at different concentrations leads to the high content production of phenols (gallic acid, cinnamic acid, caffeic acid, courmaric acid, rosmarinic acid, and chlorogenic acid) and flavonoids (quercetin, rutin, apigenin). The important compound detected in GC-MS analysis of essential oils was thymol, a monoterpene phenol (Amani *et al.*, 2020).

The inoculation of *Piriformospora indica* in the “bush pepper” of *Piper nigrum* provides significant increase in chlorophyll a and total chlorophyll content. The total oleoresin and piperine content in the berries were also increased by 10.79% and 24.29% respectively (Anith *et al*., 2018). The endophyte when accompanied with MeJA under saline conditions increased the main essential oil constituents

whereas the treatment with MeJA provided with the highest content of anthocyanin and total phenolic content in *Mentha piperita* (Khalvandi *et al*., 2019)

The effect of MeJA and SA, the concentration of the elicitor and the time of exposition on cell suspension culture of *Piper cumanense* was evaluated. The metabolic profiling of intracellular and extracellular extracts was analyzed using UHPLC- DAD and GC-MS. 100µM of SA showed the higher production of 5-hydroxymethylfurfural (6.3%), phenols (6.5%), and (Z)-9-octadecenamide (8.8%). This is the first report of elicitation on cell suspension culture in *Piper* genus (Rodríguez-Sánchez *et al*., 2020). Triterpenoides content in *Centella asiatica* was also increased under the influence of 2mM MeJA and 2mM SA by 1.4 folds and 2 folds respectively (Buraphaka & Putalun., 2020)

The effect of abiogenic elicitors on *Lycopersicum esculentum* Mill. that stimulate the innate immune response was studied and the reports suggests the most effective were 0.5% $CaCl_2$ followed with isonicotinic acid 2.5mM which after 24hrs increased the total phenolic content and total flavonoid content by 1.92 and 1.44 folds when treated with 0.5% CaCl2, with INA the content increased by 1.63 and 1.38 folds respectively (Chakraborty *et al*., 2016).

Production from endophytes

In recent decades, many reports came into light providing a parallel view or an alternate way to meet the increasing demands of the secondary metabolites i.e., with the help of endophytic fungi and bacteria. The auxenic cultures of these endophytes could act as efficient elicitors for the higher production of economically important bioactive compounds.

Various reports claimed the potential of different endophytic fungi isolated from *Piper* species to produce its principal alkaloid piperine such as *Diaporthe sps PF20, Periconia sps, Colletotricum gloeosporioides, Mycosphaerella sp. PF 13, Phomopsis sps.* Deducing the capacity to isolate and purify these compounds from the endophytes without harming the plants (Jasim *et al*., 2018; Verma *et al*., 2011; Chithra et al., 2014; Krishna *et al*., 2019; Chithra *et al*., 2017; Chithra *et al.*, 2014).

The epigentic modifiers such as valproic acid and suberohydroxamic acid increased the piperine content produced by Diaporthe sps PF20, an endophyte isolated from *Piper nigrum* by 8.7 and 3.7 folds respectively (Jasim *et al*., 2018). The inoculation of *Piriformospora indica* in black pepper

resulted in the higher produce of oleoresin and piperine content i.e., 10.75% and 24.29% respectively (Anith *et al*., 2018)

P. indica had shown substantial increase of phenolics in the hairy root cultures of the *Ficus carica* L. The 2% culture filtrate enhanced the content of gallic acid (80.5- fold), caffeic acid (26.2-fold), coumaric acid (4.5-fold), and cinnamic acid (60.1-fold), apigenin (27.6-fold) and rutin (5.7-fold) after 72hrs (Amani *et al*., 2021). A considerable increase in the piperine content is reported in *Piper nigrum* under the influence of endophytic bacteria *Pseudomonas flouresence* (Paul and Sarma., 2006).

Significance of the study:

The study will help in the better understanding of the effects of abiotic and biotic stress upon exogenous application of various elicitors in *Piper longum*. Furthermore, transcriptomic analysis will provide insight of the changes occurred on gene level due to stress in the biosynthetic pathway which will pave a way for enhancing the yield of these metabolites from molecular level.

The cultivation of *P. longum* under stressed conditions will improve the quality and quantity of the pharmacologically active constituents and this can be used to meet the increasing demands of these compounds in future.

PROPOSED METHODOLOGY

1. Establishment of *Piper longum* in the Herbal Garden, Dayalbagh Educational Institute, Agra.
2. Treatment of elicitors will be provided periodically

Chemical Elicitors: Methyl Jasmonate (MeJA) and Salicylic Acid (SA)

Biotic Elicitors: *Piriformospora indica* and *Pseudomonas flouresence*

3. Growth Parameters (Azni et al., 2021)

a) Plant Height

b) Leaf Area

c) Number Of Spikes Per Plant

d) Fresh and Dry Weight

4. Physiological Parameters: (Aftab et al., 2010)

a) Photosynthetic Rate

b) Transpiration Rate

c) Stomatal Conductance (Using Infrared Gas Analyzer) (IRGA)

d) Net Assimilation Rate.

5. Biochemical Parameters:

a) Chlorophyll Content (Lichtenthaler and Buschmann., 2001)

b) Proline Content (Bates et al., 1973)

c) Estimation of Peroxidase (Velikova et al., 2000)

d) Estimation of Superoxide Dismutase (Scebba et al., 1999)

e) Estimation of Catalase (Aebi et al., 1984)

f) Total Carbohydrates (Dubois et al., 1956)

6. Yield Parameters:

a) Total Alkaloid Content (Harborne., 1973)

b) Total Flavonoid Content (Chang et al., 2002)

c) Total Phenol Content (Kumar et al., 2021)

d) Estimation Of Piperine Content (Chatterjee et al., 2022)

a) Selected samples will be analyzed for piperine biosynthesis through Next Generation Sequencing (NGS) platform (George et al., 2022)

b) Sample collection

c) Isolation of total RNA

d) Construction of cDNA library

e) Sequencing and bioinformatic analysis

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